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ORIGINAL PAPER

Reduced vascular endothelial growth inhibitor (VEGI) expression is associated with poor prognosis in breast cancer patients

Christian Parr · Chun H. Gan · Gareth Watkins · Wen G. Jiang

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Abstract Vascular endothelial growth inhibitor (VEGI) is a novel anti-angiogenic cytokine that belongs to the tumour necrosis factor (TNF) superfamily. Very little is known about the significance of VEGI in cancer. Our study analysed VEGI expression in relation to breast cancer patient clinical parameters. The VEGI expression profile was assessed qualitatively (RT-PCR), quantitatively (real-time Quantitative-PCR), and immuno-histochemically (IHC), in a panel of 24 human normal and cancer cell lines and in a cohort of 151 mammary tissue samples (n = 33 normal breast tissue; n = 118 breast cancer tissue) with a 6-year median follow-up. Patients who had died of breast cancer or had local recurrence of the disease expressed significantly lower levels of VEGI in comparison to the elevated levels in the disease free patients. High levels of VEGI were associated with an increased chance of patient survival. Importantly, patients with breast tumours expressing reduced levels of VEGI had a poorer prognosis than those patients expressing high levels of VEGI. However, no significant correlations were observed between VEGI expression and tumour grade, TNM classification, or nodal involvement. In conclusion, VEGI is aberrantly expressed in human breast cancer tissues. VEGI displays prognostic relevance as breast cancer patients with an overall poor prognosis express significantly lower levels of VEGI compared to those with a favourable prognosis.

C. Parr (✉) · C. H. Gan · G. Watkins · W. G. Jiang
Department of Surgery, Wales College of Medicine, Metastasis and Angiogenesis Research Group, Cardiff University, Heath Park, Cardiff CF14-4XN, UK
e-mail: parrc@cf.ac.uk

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Introduction

Breast cancer is one of the leading causes of cancer death worldwide [1]. It is by far the commonest form of cancer in women, and was responsible for 27.4% of all new cancer cases, and 17.4% of all cancer-related death in European women in 2004 [2]. It has been well established that tumour development and metastasis are critically dependent upon angiogenesis, and that the degree of tumour angiogenesis correlates with breast cancer patient prognosis [3, 4]. Microvessel density (MVD) is the measurement of new blood vessel growth in and around a tumour, and is frequently used as a clinical indicator of the degree of angiogenesis. An increase in MVD is associated with an increased incidence of metastasis, and is inversely correlated with overall relapse-free survival in breast cancer patients [5–13].

Angiogenesis is tightly regulated by a network of pro-angiogenic and anti-angiogenic factors [14]. Of the known pro-angiogenic factors, vascular endothelial growth factor (VEGF) has been established as one of the most potent inducers of tumour angiogenesis, and can activate both endothelial cell proliferation and migration [15, 16]. VEGF demonstrates the ability to exert this pro-angiogenic influence in a variety of human tumours [17, 18], and reports suggest that VEGF can act also as an independent prognostic indicator for breast cancer patients [19, 20]. The tumour itself produces many of the factors that drive

angiogenesis. Therefore, the development of endogenous anti-angiogenic factors to inhibit tumour angiogenesis and other angiogenic-driven diseases may be of significant importance in cancer therapies.

Vascular endothelial growth inhibitor (VEGI), also known as tumour necrosis factor super-family, member 15 (TNFSF15) and TL1A, is a recently identified anti-angiogenic cytokine that belongs to the tumour necrosis factor (TNF) superfamily [21]. TNF family members are involved in the modulation of a wide variety of biological processes, and most notably possess anti-tumourigenic activity and are arguably the most potent inducers of cell death [22–24]. VEGI is expressed in many normal adult tissues, and is reported to be a potent endogenous inhibitor of endothelial cell growth and angiogenesis [25]. VEGI is able to interact with 2 members of the TNF receptor superfamily, these receptors are death receptor 3 (DR3) and decoy receptor 3 (DcR3) [26], but the receptor responsible for VEGI's endothelial apoptotic action is still unknown. Yang et al. [27], report that DcR3 can be used to enhance angiogenesis by blocking the autocrine angiostatic function of VEGI in human umbilical vein endothelial cells (HUVEC). However, presently very little is known about the biological role and signalling mechanisms mediating the cellular actions of VEGI, and its relevance in human cancer. We hypothesise that VEGI may have a potent influence in breast cancer. VEGI levels were assessed in a cohort of mammary tissue specimens (normal $n = 33$; cancer $n = 118$) in association with patient clinical data. The degree of VEGI expression in breast tissues may play a role in tumour pathogenesis and display correlation with patient prognosis.

Materials and methods

Cell lines and culture

All cell lines used in this study were obtained from the European Collection for Animal Cell Culture (ECACC, Porton Down, Salisbury, UK) apart from the human prostate cancer cell lines that were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, USA).

This study used human breast cancer cells (MDA-MB-157, MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MCF7, BT474, BT549, ZR-75-1), human prostate cancer cells (DU-145, PC-3, CA-HPV-10), human colorectal cancer cells (HRT-18, HT-115), human pancreatic cancer cells (MIA PACA-2), human bladder cancer cells (EJ-138, T-24), human melanoma cells (G-361), human lung carcinoma cells (A-549), human hepatocellular carcinoma cells (PLC-PRF-5), human fibroblast cells (MRC-5, Human Fibroblasts), a human epithelial cell line (ECV-304), and a human endothelial cell line (HECV).

Cells were routinely cultured with Dubecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, penicillin and streptomycin (Gibco BRC, Paisley, Scotland).

Human breast specimens

A total of 151 breast samples were obtained from breast cancer patients (33 were background normal breast tissue and 118 were breast cancer tissue). These tissues were collected immediately after mastectomy, and snap-frozen in liquid nitrogen, with approval of the local ethical committee. Background normal mammary tissues were removed from the same patients. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumour deposits. The median follow up for the cohort was 6 years (May 2001). For patient clinical data see Table 1.

Total cellular RNA preparation

Total cellular RNA was isolated from the homogenised breast samples and human cell lines using the AB Gene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, Epsom, Surrey, UK). The concentration of RNA was determined through spectrophotometric measurement (WPA UV 1101, Biotech Photometer, Cambridge, UK).

Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was prepared using 0.5 μg of the RNA sample and a reverse transcription kit (Sigma, Poole, Dorset, UK). The quality of DNA was verified using β-actin. β-actin forward and reverse primers were 5′-d(ATGATATCGCCGCGCTCGTC)-3′ and 5′-d(CGCTCG GTGAGGATCTTCA)-3′, respectively, and gave products of approximately 0.58 kb. VEGI forward and reverse primers were designed based on the human VEGI sequence (GeneBank Accession number: BD131562) 5′-d(CAAAGTCT ACAGTTTCC CAA T)-3′ and 5′-d(ACTGAACCTGACCGTACATGATT TTTAAA GTGCTGTGT G)-3′. PCR was performed in a GeneAmp PCR system 2400 thermocycler

Table 1 Breast cancer patient clinical data details

Clinical data	Grouping	Sample number
Tissue sample	Background	33
	Tumour	118
Node status	Negative	67
Node status	Positive	51
Tumour grade	1	23
	2	40
	3	55
Histological sub-types	Ductal	93
	Lobular	13
	Others	12
TNM staging	1	68
	2	39
	3	8
	4	3
Survival status	Disease free	86
	Metastases	6
	Local recurrence	9
	Died breast cancer	17
Prognosis	Good prognosis	86
	Poor prognosis	32

The patient prognosis grouping was defined so that those patients who had remained alive and disease-free were assigned to the good prognosis group, whereas, the patients who had recurrence, metastasis to another site or had died as a result of breast cancer were allocated to the poor prognosis group

(Perkin-Elmer, Norwalk CT, USA). Conditions for PCR were 40 s at 94°C, 60 s at 55°C, 60 s at 72°C (35 cycles). PCR products were loaded onto a 2% agarose gel and electrophoretically separated. The gel was then visualised under ultraviolet light following ethidium bromide staining.

Real-time quantitative polymerase chain reaction (Q-PCR)

The iCycler IQ system (BioRad, Camberley, UK) was employed [28, 29], to quantify the level (shown as copies/μl from internal standard) of VEGI in the breast specimens. Breast cDNA samples were then examined for VEGI expression using the primers described above, along with a set of standards and negative controls. The QPCR technique utilised the Amplifluor system (Intergen Inc, England) and Q-PCR master mix (ABgene, Surrey, England), in conjunction with a universal probe (UniPrimer^{TM}). Real-time QPCR conditions were 95°C for 15 min, followed by 65 cycles at 95°C for15 s, 55°C for 30 s, 72°C for 15 s. The results of the test molecules were normalised against levels of ß-actin, using a ß-actin quantitation kit from Perkin-Elmers (Perkin-Elmers, Surrey, England, UK). The epithelial content within the tumours was taken into account by normalising VEGI against cytokeratin19. CK19 forward and reverse primers were 5′-d(CAGGTCCGAGGTTACTGAC)-3′ and 5′-d(ACTGAACCTGACCGTACA CACTTTCTGCCAGTGTGTCTTC)-3′, respectively.

Western blot analysis of VEGI expression in a panel of human breast cancer cell lines

VEGI protein expression was assessed in a variety of breast cancer cell line lysates through standard Western Blot analysis. VEGI antibody was purchased from (Santa-Cruz Biotechnologies, California, USA). Protein expressed was quantified using Uvitech analysis software (Uvitech, Cambridge, UK).

Immuno-histochemical staining of breast specimens

Frozen sections of breast tumour ($n = 33$) and background tissue ($n = 33$) were cut at a thickness of 6 μm using a cryostat. The sections were mounted on super frost plus microscope slides, air-dried and then fixed in a mixture of 50% Acetone and 50% methanol. The sections were then placed in ''Optimax'' wash buffer for 5–10 min to rehydrate. Sections were incubated for 20 min in a 0.6% BSA blocking solution and probed with VEGI antibody, and without primary antibody as a negative control. Antibody to the VEGI epitope mapped to the C-terminus of human VEGI, and was purchased from Santa-Cruz Biotechnologies Inc., (Santa-Cruz, CA, USA). Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc, Glostrup, Denmark). Following washings, the Avidin Biotin Complex (Vector Laboratories, Peterborough, UK) was then applied to the sections, followed by extensive washing steps. Diamino benzidine chromogen (Vector Labs, Peterborough, UK) was then added to the sections, and incubated in the dark for 5 min. Sections were then counter stained in Gill's Haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip. Staining was independently assessed by the authors.

Statistical analysis

The results were assessed using non-paired (two-sided) Student's *t*-test and also with a Kaplan–Meyer survival curve (*P*-values by Cox Proportion Hazardous Analysis). VEGI transcript values obtained in the study are given as mean copy number ±SD. A P-value < 0.05 was defined as statistically significant.

Results

VEGI expression in human cell lines

A variety of 24 human normal and cancer cell lines were examined for the presence of VEGI mRNA transcripts through RT-PCR (Fig. 1).

VEGI was expressed in the majority of the breast cancer cell lines that are considered to be of a non/low invasive nature, as reported in previous studies [30]. The least aggressive breast cancer cell lines (MDA-MB-157, BT-474, and MCF-7) displayed the strongest level of expression. Interestingly, the more aggressive breast cancer cell lines (MDA-MB-231 MDA-MB-435S, and BT-549) expressed little or no VEGI transcripts. VEGI was found to be weakly to moderately expressed within prostate, colorectal, bladder, liver, and lung cancer cells. VEGI was also expressed within epithelial and endothelial cell lines and weakly within the stromal fibroblasts. Therefore, VEGI was found to be expressed in a wide variety of human cell lines.

VEGI protein expression in human cell lines

The VEGI protein was found to be expressed to varying degrees in the human breast cancer cell lines (see Fig. 2). A specific band at 22 kDa was identified, which corresponded to the mature VEGI protein. VEGI was found to be expressed at higher levels in the breast cancer cell lines of a low/non-invasive nature (MCF-7, MDA-MB-157, BT-474). Therefore, our Western Blot analysis confirmed the data we report with the RT-PCR.

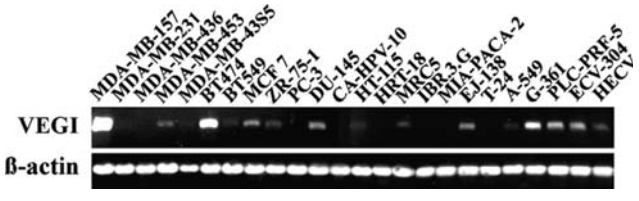

Fig. 1 RT-PCR expression of vascular endothelial growth inhibitor (VEGI) in a variety of 24 human cell lines. The expression of VEGI mRNA was assessed (and re-assessed) in a panel of human cell lines. VEGI was found to be expressed in a wide variety of human cell lines. The least aggressive breast cancer cell lines (MDA-MB-157, BT-474, and MCF-7) displayed the strongest level of expression. Interestingly, the more aggressive breast cancer cell lines (MDA-MB-231 MDA-MB-435S, and BT-549) expressed little or no VEGI transcripts

Immuno-histochemical staining of human breast specimens

VEGI immuno-staining was observed in the human breast tissue sections ($n = 33$ pairs). VEGI was expressed in the normal mammary tissue (Fig. 3 – left panel), and was mainly confined to the epithelial cells, which revealed intense cytoplasmic staining for VEGI. It was also noted that selective stromal cells also displayed a weak staining for VEGI. However, most stromal cells were negative for VEGI. We report that VEGI protein levels were dramatically reduced/absent in the cancer cells from in the tumour tissue samples (Fig. 3 – right panel). Stromal cells in tumour tissues were also negative for VEGI expression. Finally, no obvious endothelial cell staining was observed in either normal or tumour tissues.

Real-time quantitative-PCR analysis of VEGI expression in human breast cancer specimens

We quantified the mean VEGI transcript expression in the breast specimens (tumour $n = 118$, background $n = 33$) using real-time Q-PCR (all values are displayed as mean VEGI transcript copies/µl of RNA with 50 ng total RNA). We report that there appeared to be no significant difference between the normal background tissues (72.4 ± 25.8) and the tumour tissues (99.4 ± 18.7) (Fig. 4a).

VEGI expression and node status

We assessed the degree of VEGI expression in relation to the node status of the breast cancer patients (patients with negative nodes $n = 67$; patients with positive nodes $n = 51$). We report that patients without node involvement (104.6 ± 18) had no significant differences in VEGI expression compared to those patients with node involvement (89.5 ± 24) (Fig. 4b).

VEGI and breast tumour grade

There was no apparent difference in VEGI transcript levels between the well differentiated Grade 1 tumours (68.5 ± 25, $n = 23$), the moderately differentiated Grade 2 classed tumours (122 ± 22, $n = 40$), and the poorly differentiated Grade 3 tumours (97.5 ± 17.2, $n = 55$) (Fig. 3c). We also examined VEGI expression within the varying tumour histological subtypes, and report that VEGI levels were reduced, although not statistically ($P = 0.095$) within lobular tumours (31 ± 5.7), compared to the ductal tumours (111 ± 15) (Fig. 4d).

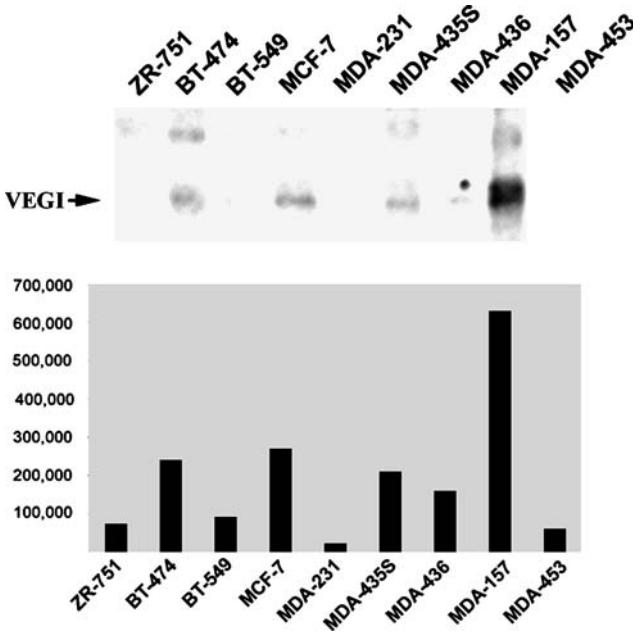

Fig. 2 Protein expression of vascular endothelial growth inhibitor (VEGI) in a variety of breast cancer cell lines. Western blotting (upper panel) was used to assess VEGI protein expression in a variety of human breast cancer cell lines. VEGI protein levels were quantified using Uvitech analysis software (lower panel). VEGI was expressed to a higher degree in the breast cancer cell lines of a non/low invasive nature (MDA-MB-157, BT-474, and MCF-7), compared to the more aggressive cell lines (MDA-MB-231 and BT-549)

Tumour-node-metastasis classification of patients

VEGI expression did not appear to correlate with patient outlook through tumour-node-metastasis (TNM) grouping (TNM 1, $n = 68$; TNM 2, $n = 39$; TNM 3, $n = 8$; TNM 4, $n = 3$) (Fig. 4e). Levels of VEGI appeared to be dramatically reduced in patients with a very poor outcome TNM 4 group (16.7 ± 4.3), compared with patients from TNM 1 (100 ± 16), TNM 2 (114 ± 25) and TNM 3 (108 ± 46), however, the sample was too small and did not meet the criteria to allow for statistical significance.

VEGI expression and survival status

We assessed the survival status of the breast cancer patients in association with VEGI expression, with a 6-year median follow-up period (Fig. 4f). It was discovered that breast cancer patients who had died of breast cancer (46.2 ± 22, $n = 17$), or that now had local recurrence (21.8 ± 16, $n = 9$), had significantly lower levels of VEGI ($P = 0.0028$ and $P = 0.038$, respectively) expressed by their breast tumours, compared to those patients who were still disease-free (102 ± 14, $n = 86$), and those that had metastases at another site in the body (117 ± 70, $n = 6$).

VEGI expression in relation to patient prognosis

Patients were divided into two groups, those who had remained alive and disease-free were assigned to the good prognosis group ($n = 86$), whereas, the patients who had recurrence, metastasis to another site or had died as a result of breast cancer were allocated to the poor prognosis group ($n = 32$). The quantity of VEGI from each tumour specimen was assessed and we reveal that the patients with a poor prognosis had significantly reduced levels of VEGI ($P = 0.048$), compared to the patient samples with a good prognosis. Our results show that the good prognosis group had elevated levels of VEGI (102 ± 14), whereas those patients with a poor prognosis had reduced levels of VEGI (69.5 ± 21) (Fig. 4g).

In addition, we also examined the breast tumour VEGI expression levels in comparison with the number of surviving patients using a Kaplan–Meier Survival Curve. The number of surviving breast cancer patients (Cum. Survival), was measured against the number of months post surgery. VEGI values above 1 were classed as high for the mean copy number values.

These results demonstrate that the degree of VEGI expression may be relevant in terms of breast cancer patient survival. A low level of VEGI was associated with a poorer chance of survival, whereas, a high level of VEGI expression correlated to an increased level of survival (Fig. 4h). These results however, did not reach statistical significance using Cox Proportion Hazardous Analysis.

Discussion

It has been well documented that angiogenesis is one of the most crucial steps facilitating tumour growth [31, 32]. Angiogenesis helps to transport oxygen and nutrients to the tumour, which is essential for tumour development. During the metastatic cascade, tumour cells will attempt to migrate and invade through tissues to reach the vasculature in order to continue their route through the metastatic process. In a number of studies it has been shown that tumour angiogenesis is correlated with tumour malignancy and patient prognosis [33].

The process of angiogenesis is tightly regulated by a balance of pro- and anti-angiogenic molecules [34]. Development of endogenous anti-angiogenic factors that inhibit tumour neovascularisation and other angiogenesis-driven diseases may be of significant importance. A novel cytokine of the TNF superfamily, VEGI, has been identified as a highly effective

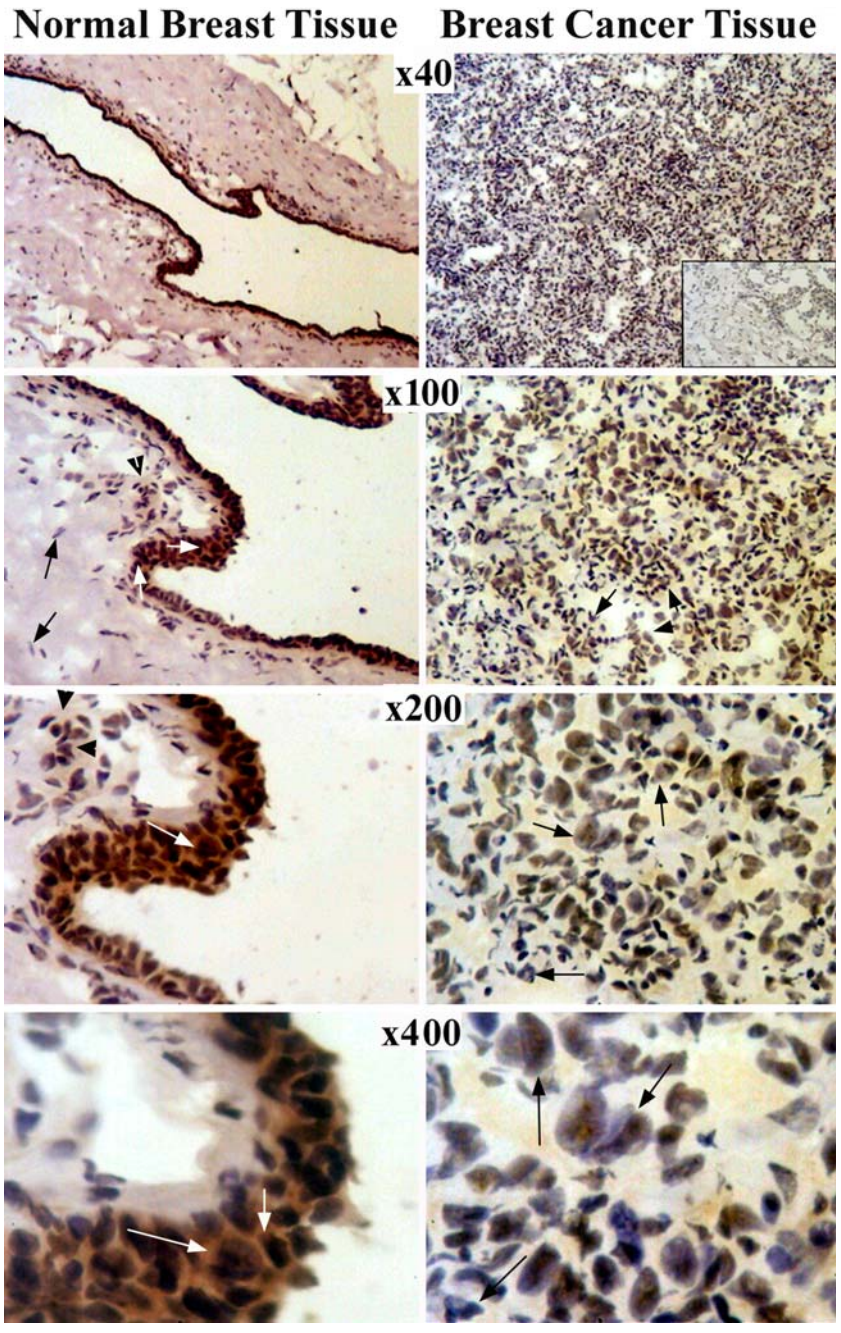

Fig. 3 Immuno-histochemical staining of human breast specimens. Left panel: Normal background mammary tissue. The vascular endothelial growth inhibitor (VEGI) protein was found to be well stained in the cytoplasmic area of normal breast epithelial cells (indicated by white arrows), and absent (black arrows) or weakly expressed (black arrow-heads) in the stromal fibroblasts. Right panel: Breast tumour tissue. Staining was dramatically reduced in the breast tumour tissues. VEGI expression was seen to be negative or very weakly positive in the cancer cells of breast cancer patients (black arrows). No obvious endothelial cell staining was observed in either normal or tumours tissues. Breast cancer tissues were also stained in the absence of a VEGI primary antibody to act as a negative control (top right insert)

inhibitor of angiogenesis [21, 25]. This group also report that VEGI enforces two distinctly different actions on endothelial cells, the growth arrest of G_0–G_1 cells and apoptosis of proliferating cells, although both these actions result in the same goal: suppression of the angiogenic drive [35]. A recent study demonstrated that an isoform of VEGI (VEGI-192), was able to eliminate tumour vascular endothelial cells, which resulted in suppression of tumour growth in a Lewis lung cancer murine tumour model [36]. However, the full-length version of VEGI (VEGI-251) is the most abundant isoform, and is reported to be an endothelial cell-secreted inhibitor of angiogenesis that retarded human xenograft tumour growth in vivo [37]. However, very little is known about the significance of VEGI in human cancer. Our study assessed VEGI levels quantitatively, immuno-histochemically (IHC) and qualitatively in a cohort of breast cancer patient specimens compared with background breast tissue. This study is the first to analyse VEGI expression in cancer patients in relation to patient clinical parameters. We demonstrate that VEGI transcripts are

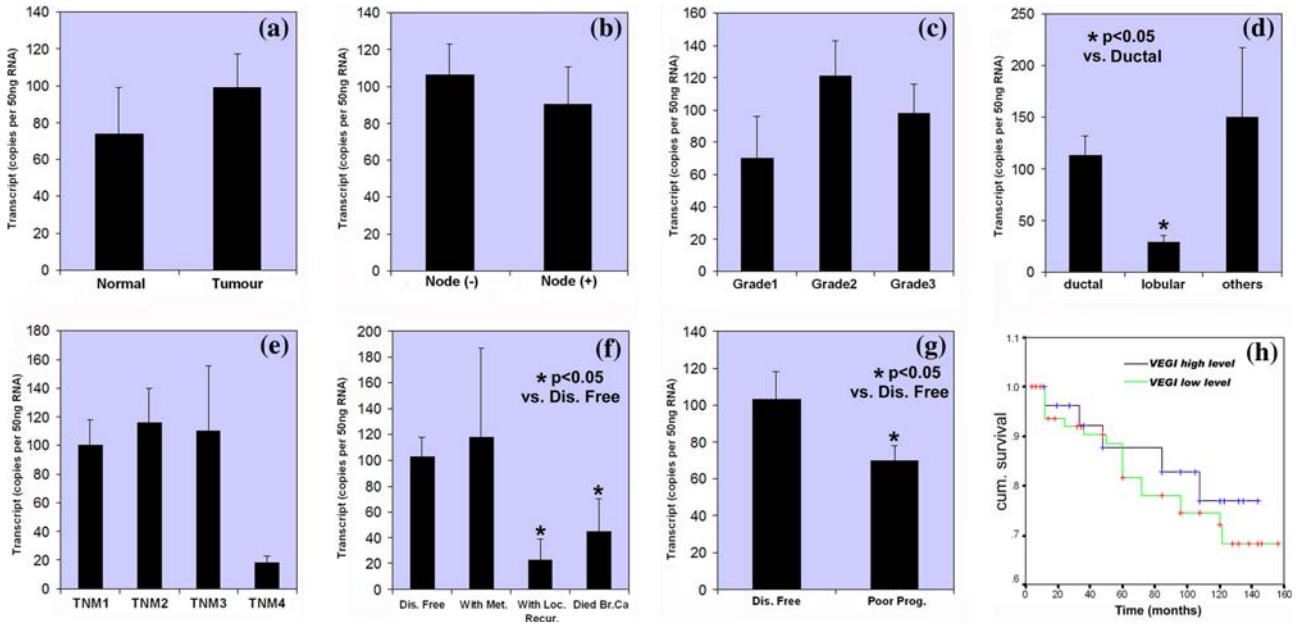

Fig. 4 Quantitative-PCR Analysis of vascular endothelial growth inhibitor (VEGI) expression in human breast cancer tissues. (**a**) Tumour versus background tissue from breast cancer patients; (**b**) node negative versus node positive patients; (**c**) tumour grade; (**d**) tumour histological subtypes; (**e**) tumour-node-metastasis classification; (**f**) survival status; (**g**) patient prognosis; (**h**) Kaplan–Meier survival analysis

aberrantly expressed in human breast cancer tissues and correlate with overall patient prognosis.

Our studies revealed that VEGI mRNA was expressed in a wide variety of human cancer cell lines, including breast, prostate, bladder, colorectal and liver. VEGI was also expressed in human epithelial cells, therefore suggesting a normal physiological role for the molecule in maintaining a stable vasculature. In the breast cancer cell lines, VEGI mRNA and protein was found to be expressed to higher levels within the cell lines that are generally considered to be of a less aggressive and non-invasive nature. Interestingly, VEGI was found to be absent or expressed at low levels within the more aggressive/invasive breast cancer cell lines. VEGI is a type II transmembrane protein, therefore, VEGI may be ideally situated on the cell membrane to play a role in governing the aggressive/invasive properties of breast cancer cells. Three isoforms of VEGI have so far been reported, only the secreted soluble form displays anti-angiogenic properties [21, 35, 37], and therefore maybe the cell-associated form may serve another purpose. VEGF has the ability to induce cell proliferation or migration, so in contrast maybe the presence of VEGI can suppress or limit cancer cell migration and invasion. Thus far, studies have shown that VEGI can suppress endothelial cell growth [21], however its reported growth influence on other cell types have been inconsistent.

This study demonstrates, through IHC staining, that VEGI was strongly expressed in the epithelial cells of normal background breast tissue of breast cancer patients; however, VEGI levels were dramatically reduced in the tumour tissue of patients. Our study also shows that VEGI staining in stromal cells of mammary tissues was either negative or weakly positive. Taken together with data from cell lines, which revealed negative (IBR3G) to weakly positive (MRC5) VEGI levels in fibroblasts, it is suggested that VEGI is primarily of epithelial origin in tissues. In addition, VEGI levels tended to be lowest in lobular tumours compared to tumours of ductal origin. The reduction of VEGI expression observed in the tumour tissues may shift the balance of angiogenesis to favour pro-angiogenic conditions.

Comparison of patient clinical data with tumour VEGI levels revealed that VEGI may represent a survival factor in human breast cancer. Our quantitative study demonstrated that VEGI expression correlated with breast cancer patient prognosis, as a high degree of VEGI expression was associated with an overall good prognosis and increased survival time. These results may be explained by the anti-angiogenic nature of VEGI. VEGI may serve as a normal physiological regulator of angiogenesis, therefore a high VEGI level in breast tumours may limit/suppress angiogenic activity, and thus the life-threatening potential of the tumour may be reduced. In contrast,

we report that decreased VEGI levels in patient tumour specimens, was associated with patient death as a result of breast cancer, local recurrence, decreased survival time and an overall poorer prognosis for the patient. This absence/reduction of tumour VEGI expression suggests that there may have been a shift in the balance between pro- and anti-angiogenic stimuli. A reduction in VEGI expression favours pro-angiogenic conditions, which may result in autonomous vascular growth. This loss of regulation may have subsequently produced an environment conducive to tumour growth and survival, resulting in a poorer prognosis for the breast cancer patient. However, it must also be mentioned that no significant correlations were observed between VEGI expression and tumour grade, TNM classification, or nodal involvement.

Our current knowledge of the regulatory mechanisms balancing the angiogenic versus angiostatic drive remain poorly understood, however this report suggests that VEGI may act as useful anti-angiogenic agent that also displays potential as an indictor of breast cancer patient prognosis. A recent study suggested that VEGI expression is directly under the control of TNF-α receptor-mediated signalling, as treatment with the pro-inflammatory cytokine TNF-α induced the expression of VEGI in articular chondrocytes and cerebral endothermic cells [38, 39]. However, the mechanism behind VEGI anti-angiogenic signalling is unclear; therefore, any molecular targets need to be identified to get a clearer understanding of the full potential of this novel cytokine.

This is the first study to examine the role of VEGI in breast cancer. We conclude that there are aberrant levels of VEGI expressed in human breast cancer tissues, and demonstrate that VEGI displays prognostic value. Breast cancer patients with an overall poor prognosis expressed significantly lower levels of VEGI compared to those with a favourable prognosis. We also suggest that VEGI demonstrates the potential to become an exciting anti-angiogenic molecule with therapeutic potential.

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